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# Biosynthesis of glycosyl-phosphatidylinositol lipids in *Trypanosoma brucei:* involvement of mannosyl-phosphoryldolichol as the mannose donor

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**Trypanosome variant surface glycoproteins (VSGs) exemplify a class of eukaryotic cell-surface glycoproteins that rely on a covalently attached lipid, glycosyl-phosphatidylinositol, for membrane attachment. The glycolipid anchor is acquired soon after translation of the polypeptide, apparently by replacement of a short sequence of carboxyl-terminal amino acids with a precursor glycolipid. A candidate glycolipid precursor (P2) and a related glycolipid (P3) have been identified in polar lipid extracts from trypanosomes. Both lipids are glycosylphosphatidylinositol species containing a $Man_3GlcN$ core glycan indistinguishable from the backbone sequence of the VSG glycolipid anchor. We and others have recently described the cell-free synthesis of P2, P3, and a spectrum of putative biosynthetic lipid intermediates using crude preparations of trypanosome membranes. In this paper we use these preparations to show that all three mannose residues in the glycosyl-phosphatidylinositol glycan are derived from dolichol-P-mannose.**

*Key words:* dolichol/glycoprotein/membrane anchor/glycosyl-phosphatidylinositol/trypanosome

## Introduction

A variety of proteins are attached to eukaryotic cell surfaces via inositol-containing glycophospholipids (GPIs). Current models suggest that the glycolipid anchors are first synthesized as precursor glycolipids which are then covalently coupled to target proteins in the endoplasmic reticulum (ER). The entire process occurs soon (1-5 min) after completion of protein synthesis, presumably after translocation of the polypeptide into the lumen of the ER. In all cases where data are available, the lipid anchor replaces a short sequence of mainly hydrophobic amino acids at the carboxyl terminus of the protein (reviewed by Ferguson and Williams, 1988; Cross, 1990).

Much of the information on the structure and biosynthesis of glycolipid membrane anchors has come from studies of the variant surface glycoproteins (VSGs) and GPIs of the parasitic protozoan, *Trypanosoma brucei*. The glycolipid anchor of one VSG (VSG 117) has been completely chemically defined (Ferguson *et al.*, 1988) and consists of the backbone sequence ethanolamine-phosphate-6Man$\alpha$1-2Man$\alpha$1-6Man$\alpha$1-4GlcN$\alpha$1-6inositol (Figure 1). The inositol residue is linked via a phosphodiester bond to dimyristylglycerol, the ethanolamine group is amide-linked to the carboxyl terminal amino acid of the mature protein and a galactose side-chain is attached to the mannose residue adjacent to glucosamine (Figure 1). Similar backbone structures with different side-chain modifications and lipophilic groups have been described for the lipid anchors of rat brain Thy-1 (Homans *et al.*, 1988) and human erythrocyte acetylcholinesterase (Roberts *et al.*, 1988a,b).

A candidate glycolipid anchor precursor, P2 [Menon *et al.*, 1988a; presumably identical to (glyco)lipid A, Krakow *et al.*, 1986], and a related glycolipid, P3 [Menon *et al.*, 1988a; presumably identical to (glyco)lipid C, Krakow *et al.*, 1986], have been identified in *Trypanosoma brucei*. Both lipids are glycosyl-phosphatidylinositol (GPI) species containing a single N-unsubstituted ethanolamine residue, a $Man_3GlcN$ core glycan indistinguishable from the backbone sequence of the VSG glycolipid anchor, and a dimyristyl-phosphatidylinositol moiety (Menon *et al.*, 1988a; Mayor *et al.*, 1990a,b). P2, like the VSG glycolipid anchor, is susceptible to cleavage by phosphatidylinositol specific phospholipase C (PI-PLC), whilst fatty acids esterified to one (or more) of the inositol hydroxyl groups in P3 render P3 insusceptible to the enzyme (Krakow *et al.*, 1989; Mayor *et al.*, 1990b). The structures of the two lipids are shown in Figure 1.

Considerable progress has been made recently in establishing the pathway of GPI biosynthesis. The glycolipid precursors and a spectrum of putative biosynthetic intermediates have been synthesized *in vitro* by incubating radiolabelled sugar nucleotides with crude preparations of trypanosome membranes (Menon *et al.*, 1988b; Doering *et al.*, 1989; Masterson *et al.*, 1989; Menon *et al.*, 1990). Structural analyses of the *in vitro* labelled lipids (Masterson *et al.*, 1989; Menon *et al.*, 1990) suggest that GPI species are most probably assembled by sequential monosaccharide addition to PI. Assembly is initiated by the transfer of GlcNAc from UDP-GlcNAc to PI followed by the deacetylation of GlcNAc-PI to give GlcN-PI (Doering *et al.*, 1989). The glycolipid then appears to be built up by the stepwise addition of three mannose residues and the terminal ethanolamine phosphate group (reviewed by Doering *et al.*, 1990). An unusual feature of trypanosome GPI assembly *in vitro* is that the products appear to be heterogeneously acylated forms of P2 and P3 which are converted to dimyristyl species via a series of fatty acid re-modelling reactions (Masterson *et al.*, 1989, 1990). Possible roles for inositol acylation in GPI assembly and re-modelling have been postulated but remain to be established (Menon *et al.*, 1990; Masterson *et al.*, 1990).

Two separate lines of evidence suggest that one or more of the core mannose residues in the GPI glycan is derived from a lipid-linked mannose donor, dolichol-P-mannose. The first piece of evidence is based on studies of a murine T-cell lymphoma mutant (Thy-1$^-$ class E; Hyman, 1985) selected

for its inability to express the Thy-1 glycoprotein on the cell surface and subsequently shown to be defective in the synthesis of dolichol-P-mannose (Chapman *et al.*, 1980). This enzymatic defect results in the accumulation of truncated dolichol-linked oligosaccharide intermediates with five rather than nine mannose residues (Trowbridge and Hyman, 1979; Chapman *et al.*, 1979; 1980) and the synthesis of proteins with initially small ($Glc_3Man_5GlcNAc_2$) asparagine-linked oligosaccharides. More recent work has demonstrated the inability of the class E mutant cells to synthesize the Thy-1 glycolipid anchor (Conzelmann *et al.*, 1986; Fatemi and Tartakoff, 1986; Fatemi *et al.*, 1987), thus providing circumstantial evidence for the involvement of dolichol-P-mannose in anchor biosynthesis.

The second piece of evidence is derived from experiments with 2-fluoro-2-deoxy-glucose (FGlc), an inhibitor of dolichol-P-mannose synthesis that has been previously used to reproduce the Thy-1 class E mutant pattern of truncated dolichol-linked oligosaccharides in normal chick embryo fibroblasts (Datema *et al.*, 1980). We have recently shown that the incorporation of [$^3$H]ethanolamine, [$^3$H]glucosamine and [$^3$H]mannose into the glycolipid anchor precursors P2 and P3 is considerably inhibited in FGlc-treated trypanosomes (Schwarz *et al.*, 1989), consistent with a role for dolichol-P-mannose in P2 and P3 synthesis. The apparent accumulation of a non-mannosylated species, GlcN-PI, in the FGlc-treated cells further suggests that dolichol-P-mannose is involved in the addition of the first mannose residue.

Here we use cell-free systems capable of synthesizing glycosyl-phosphatidylinositol lipids to show that all three mannose residues in the GPI glycan are derived from dolichol-P-mannose.

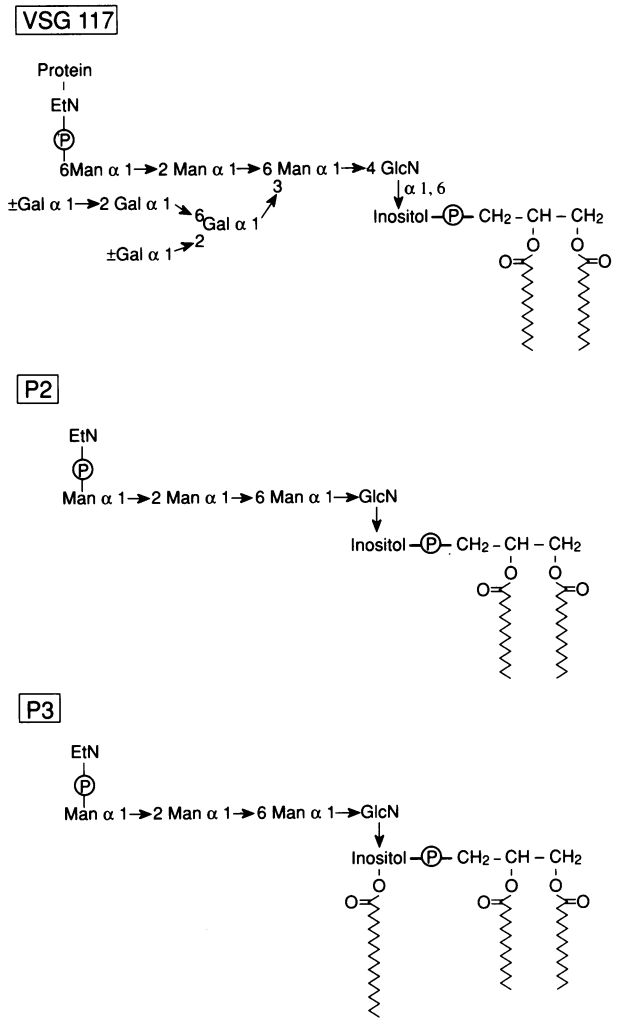


**Fig. 1.** Structures of the VSG 117 glycolipid anchor and of the glycolipids P2 and P3. The structures are taken from Ferguson *et al.* (1988), Menon *et al.* (1988) and Mayor *et al.* (1990a,b). The galactose side-chain of the VSG 117 glycolipid anchor depicted in the figure is absent from the anchor of VSG 118 (Mayor *et al.*, 1990a). The linkages not explicitly described in the structures of P2 and P3 are likely to be EtN-P-6Man, Manα1-4GlcN and GlcNα1-6Inositol, identical to the structure of the VSG glycolipid anchor. The diacylglycerol moiety in all three structures appears to be dimyristylglycerol. Dephosphorylation of P2 and P3 by treatment with ice-cold 50% aqueous HF for 60 h results in the formation of a water-soluble glycan ($Man_3GlcN$-Inositol) from P2 and a lipophilic glycan ($Man_3GlcN$-acyl inositol) from P3. The P3 glycan can be rendered water-soluble (and consequently identical to the P2 glycan) by treatment with mild alkali. The differential solubility of inositol- and acyl inositol-linked glycans has been previously exploited to define the components of lipid III (see Table I). EtN, ethanolamine; Man, mannose; GlcN, glucosamine; Gal, galactose; P, phosphate.

## Results

The lipids referred to in the text are listed in Table I and the structures of the glycolipids P2 and P3 are shown in Figure 1. Lipids I, II, IIIA, IIIB and IV are candidate biosynthetic intermediates in the assembly of P2 and P3. The dolichol moiety (trypanosome dolichol, $dol_t$) in lipid VI ($dol_t$-P-mannose) contains 11 or 12 isoprene units (P.Löw, S.Mayor, A.K.Menon, unpublished data) in contrast to the mammalian dolichols ($dol_m$) which are more hydrophobic and contain 16–23 isoprene units (Chojnacki and Dallner, 1989).

The biosynthetic intermediates (lipids I–IV), along with lipid VI, are quantitatively recovered from labelled membranes by extraction with chloroform/methanol(CM, 2:1, v/v). The mature ethanolamine-phosphate-containing

**Table I.** [$^3$H]Mannose labelled lipids synthesized *in vitro*

| Mannosylated lipid | Structure |
|---|---|
| P2 | EtN-P-$Man_3GlcN$-PI |
| P3 | EtN-P-$Man_3GlcN$-acyl PI |
| Lyso P2 | EtN-P-$Man_3GlcN$-lyso PI |
| Lipid I | $Man_3GlcN$-PI |
| Lipid II | $Man_2GlcN$-PI |
| Lipid IIIA | $Man_1GlcN$-PI |
| Lipid IIIB | $Man_3GlcN$-acyl PI |
| Lipid IV | $Man_2GlcN$-acyl PI |
| Lipid VI | $Dol_t$-P-Man |
| P1 | $Dol_t$-PP-$GlcNAc_2Man_5$ |

The table summarizes data from Menon *et al.* (1990) and Masterson *et al.* (1989). The nomenclature is similar to that used by Menon *et al.* (1990). The lipids were labelled by incubating 100 000 *g* membranes or washed preparations of lysed trypanosomes with GDP-[$^3$H]mannose as described in Materials and methods. Fatty acid remodelling reactions result in at least three forms of P2 differing in fatty acid composition (Masterson *et al.*, 1989, 1990); mature P2 contains only myristic acid. Lipids IIIA and IIIB co-chromatograph in two TLC systems (silica 60, solvent systems A and B) as lipid III. The abbreviations used are as follows. EtN, ethanolamine; Man, mannose; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; PI, phosphatidylinositol; $Dol_t$, trypanosome dolichols containing primarily 11 and 12 isoprene units; P, phosphate; PP, pyrophosphate.

glycolipids, P2 and P3, are solubilized in a second extraction step using chloroform/methanol/water (CMW, 10:10:3, v/v/v). If, as in some experiments, the initial CM extract is omitted, *all* the lipids are recovered in the CMW extract. Water-soluble contaminants in the CMW extract are removed by drying and phase separation between water and *n*-butanol: the glycolipids are recovered in the butanol phase (termed 'CMW-derived butanol extract').

Two types of membrane preparations ('lysates' and 'membranes') are used in the experiments described below. The term 'lysate' will be used to refer to washed preparations of lysed trypanosomes prepared as described by Masterson *et al.* (1989); 'membranes' will refer to the 100 000 *g* pellet obtained after differential centrifugation of lysed and homogenized trypanosomes as described by Menon *et al.* (1990).

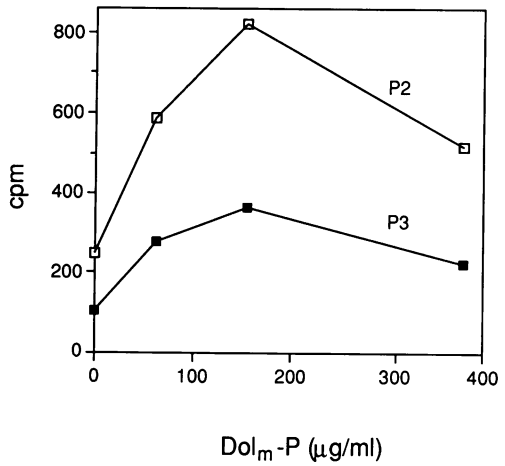


**Fig. 2.** Dolichol-phosphate stimulates [$^3$H]mannose incorporation into P2 and P3. Pig liver dolichol phosphate (dol$_m$-P) was added to incubations of membranes (50 μl, 1.5 × 10$^9$ trypanosome equivalents) with GDP-[$^3$H]mannose (1.5 μCi). After incubation at 37°C for 30 min, the membranes were extracted with CM and CMW and the CMW-derived butanol phases were analysed by TLC (silica 50000 HPTLC, solvent system D). Radioactivity was detected with a Berthold LB 2842 automatic scanner and quantitated by using the integration software supplied with the scanner in conjunction with liquid scintillation counting. The points represent the mean value of duplicate determinations at each concentration of dol$_m$-P; the range of the duplicates was ~5%.

### *Dolichol phosphate stimulates [$^3$H]mannose incorporation into P2 and P3*

Figure 2 shows that the addition of pig liver dolichol phosphate (dol$_m$-P) to incubations of membranes with GDP-[$^3$H]mannose results in an almost 4-fold stimulation of [$^3$H]mannose incorporation into P2 and P3 at the optimal dose (~0.15 mg/ml dol$_m$-P). Examination of the CM extract from dol$_m$-P stimulated, GDP-[$^3$H]mannose labelled membranes showed a major peak corresponding to dol$_m$-P-mannose, migrating ahead of trypanosome dol$_t$-P-mannose and other previously identified lipids (data not shown). In the presence of dol$_m$-P (0.15 mg/ml), incorporation of [$^3$H]mannose into lipid III increased ~3.5-fold and labelling of dol$_t$-P-mannose decreased slightly (~1.5-fold). Although a physico-chemical explanation for the dol$_m$-P stimulation result cannot be ruled out, these data suggest the involvement of dol-P-mannose as a mannose donor in glycosyl-PI synthesis.

The results also indicate that trypanosomes contain a mannosyl-transferase activity capable of transferring mannose from GDP-mannose to long chain dol$_m$-P species as well as to endogenous dol$_t$-P. This observation is consistent with earlier work on yeast GDP-Man: dolichyl phosphate mannosyl-transferase which indicated that $C_{100}$ and $C_{55}$ dolichol phosphates (20 and 11 isoprene units) were equally good substrates for the enzyme (Palamarczyk *et al.*, 1980).

### *Amphomycin inhibits in vitro synthesis of P2 and P3 and mannosylated biosynthetic intermediates*

The dol-P-mannose stimulation results were substantiated by inhibitor studies using amphomycin, a lipopeptide antibiotic known to inhibit the enzymatic transfer of mannose, glucose and GlcNAc-1-P from their nucleotide derivatives to dol-P (Kang *et al.*, 1978; Banerjee *et al.*,1981; Elbein, 1987; Banerjee, 1989). The antibiotic forms a complex with dol-P thereby preventing the synthesis of dol-P-man, dol-P-Glc and dol-PP-GlcNAc (Banerjee, 1989; Banerjee *et al.*, 1981).

**Table II.** Effect of amphomycin on [$^3$H]mannose incorporation into lipids[a]

| Expt | Assay | Amphomycin[b] (mg/ml) | Dol$_m$-P[c] (mg/ml) | P1 ← | Lyso P2 | P2 | P3 | Lipid III c.p.m. × 10$^{-3}$ | Lipid II | Lipid I | Lipid VI | Dol$_m$-P-M[e] → |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1 | 0 | 0 | ND[d] | ND | 2.24 | 0.61 | ND | ND | ND | ND | – |
| | 2 | 0.17 | 0 | ND | ND | 1.47 | 0.51 | ND | ND | ND | ND | – |
| | 3 | 0.42 | 0 | ND | ND | 0.17 | 0.12 | ND | ND | ND | ND | – |
| B | 1 | 0 | 0 | 3.03 | 13.8 | 3.27 | 6.42 | 61.8 | 16.3 | 18.9 | 44.0 | – |
| | 2 | 0.59 | 0 | 3.02 | 0.08 | 0.10 | 0.45 | 0.25 | 1.38 | 0.06 | 1.0 | – |
| C | 1 | 0 | 0 | ND | ND | 0.64 | 0.57 | 16.2 | ND | ND | 197 | – |
| | 2 | 0.31 | 0 | ND | ND | 0.08 | 0.06 | 0.80 | ND | ND | 4.65 | – |
| | 3 | 0 | 0.16 | ND | ND | 1.31 | 0.52 | 56.3 | ND | ND | 125 | 717 |
| | 4 | 0.31 | 0.15 | ND | ND | 0.91 | 0.47 | 22.9 | ND | ND | 61.8 | 243 |
| | 5 | 0.76 | 0 | ND | ND | 0.05 | 0.05 | 0.07 | ND | ND | 0.16 | – |
| | 6 | 0.73 | 0.15 | ND | ND | 0.06 | 0.06 | 0.27 | ND | ND | 0.60 | 0.64 |

[a]The lipids are defined in Table I; Dol$_m$-P is pig liver dolichol phosphate
[b]Added as a 10 mg/ml suspension in water
[c]Added as a 4 mg/ml solution in 0.2% Triton X-100 (final concentration of detergent < 0.02%)
[d]ND: not determined
[e]Dol$_m$-P-mannose: not synthesized in the absence of Dol$_m$-P

The inhibitory effect on the synthesis of all three glycolipids can be overcome by exogenous dol-P (Banerjee *et al.*, 1981).

Membranes were incubated with GDP-[$^3$H]mannose in the presence of amphomycin and [$^3$H]mannose labelled lipids were extracted and analysed by TLC. Preliminary experiments (Table II, A1−3) showed that amphomycin (0.42 mg/ml) reduced the incorporation of [$^3$H]mannose into P2 and P3 by ~92% and ~80% respectively. More detailed analyses showed that the antibiotic affected the *in vitro* synthesis of the entire spectrum of previously identified mannosylated biosynthetic intermediates: in the presence of 0.59 mg/ml amphomycin, [$^3$H]mannose incorporation into lyso P2, P2, P3 and lipids I−III was inhibited by 92−99% and the synthesis of $dol_t$-P-[$^3$H]mannose was reduced by 98% (Table II, B1−2). Similar results were obtained in experiments with washed preparations of lysed trypanosomes ('lysates') prepared as described by Masterson *et al.* (1989) (Table IV, compare B and C; Figure 4, compare panels B and C). Since the glycolipids are probably assembled by sequential addition of mannose residues (Masterson *et al.*, 1989; Menon *et al.*, 1990), the data suggest that at least the first mannose residue (i.e. proximal to the glucosamine residue) in P2 and P3 is derived from dol-P-man.

The amphomycin dependent inhibition of [$^3$H]mannose incorporation into glycosyl-PI species and into dol-P-mannose could be overcome by exogenous $dol_m$-P in accordance with the mechanism of action of the antibiotic described above. The pattern of inhibition seen in the presence of amphomycin (0.3 mg/ml, Table II, C1−2) was reversed when 0.16 mg/ml $dol_m$-P was included in the assay mixture (Table II, C4), consistent with the recovery of significant quantities of labelled $dol_m$-P-[$^3$H]mannose and $dol_t$-P-[$^3$H]mannose (Table II, C1−4). The same concentration of $dol_m$-P was too low to rescue dol-P-mannose synthesis in the presence of a higher dose (0.73 mg/ml) of amphomycin.

In contrast to the significant inhibition of dol-P-man and glycosyl-PI synthesis, amphomycin (0.59 mg/ml, Table II, B1−2) did not affect the synthesis of [$^3$H]mannose labelled P1 ($dol_t$-PP-$GlcNAc_2Man_5$) (S.Mayor, A.K.Menon, G.A.M.Cross, P.Löw, I.K.Vijay, M.A.J.Ferguson, R.A.Dwek and T.W.Rademacher, unpublished observations). Mannosylation steps in P1 assembly are expected to be insensitive to amphomycin since all five mannose residues in the P1 glycan are likely to be derived directly from GDP-mannose (Kornfeld and Kornfeld, 1985). However, the amphomycin dependent entrapment of dol-P would be expected to affect the first step in the *de novo* synthesis of P1, the transfer of GlcNAc-1-P from UDP-GlcNAc to dol-P. The data suggest that under the experimental conditions used, most of the [$^3$H]mannose labelled P1 is synthesized by the addition of [$^3$H]mannose and GlcNAc to existing pools of $dol_t$-PP-$GlcNAc_{1-2}$.

### *Pulse−chase labelling of P2 and P3 in vitro: GDP-mannose is not the immediate precursor of the mannose residues in the two glycolipids*

The synthesis of [$^3$H]mannose labelled P2 and P3 via GDP-[$^3$H]mannose *in vitro* is enhanced in the presence of UDP-GlcNAc. In order to make the synthesis totally dependent on added UDP-GlcNAc, lysates were incubated with 0.3 μM GDP-mannose for 20 min at 37°C to deplete endogenous GlcN-PI. Pre-incubated lysates were then labelled with GDP-[$^3$H]mannose for 10 min at 37°C. Table IIIA and Figure 3A show that relatively little radioactivity is incorporated into P2, P3 and other GPI lipids under these conditions, and that the major labelled product is $dol_t$-P-mannose. Two minor species running in the vicinity of lipid I could not be identified on the basis of TLC mobility: these may be dolichol-linked oligosaccharides synthesized from residual lipid precursors in the lysates since *de novo* synthesis of these molecules is inhibited in the presence of tunicamycin (S.Mayor, A.K.Menon and G.A.M.Cross, in preparation).

The labelling protocol described above was used in pulse−chase experiments to assess the role of GDP-mannose in GPI assembly. Pulse-labelled lysates were chased with non-radioactive GDP-mannose (1 mM) in the presence or absence of UDP-GlcNAc (1 mM). The chase incubations were stopped at the indicated times and lipids were extracted into CMW. The CMW extracts were then dried and partitioned between water and butanol, and the butanol phases were analysed by TLC (Figure 3). The data are summarized in Table III.

After a 60 min chase in the absence of UDP-GlcNAc, the spectrum and intensity of [$^3$H]mannose labelled GPI lipids was essentially identical to that obtained at the end of the

**Table III.** Pulse−chase labelling of P2 and P3 *in vitro*

| Sample | Chase time (min) | UDP-GlcNAc in chase | P2 | P3 | Mannosylated intermediates[b] (c.p.m.) | $dol_t$-P-man[c] |
|---|---|---|---|---|---|---|
| A | 0 | − | 430 | 200 | 1500 | 70900 |
| B[d] | 30 | + | 4980 | 1980 | 1735 | 5095 |
| C[d] | 60 | + | 3325 | 1145 | 1690 | 3620 |
| D | 60 | − | 115 | 155 | 1635 | 6385 |

Lysates were pre-incubated at 37°C, pulse-labelled with GDP-[$^3$H]mannose, and chased in the presence or absence of UDP-GlcNAc[a]. Reactions were stopped by adding ice-cold incubation buffer to give a final volume of 250 μl, followed by 1.7 ml ice-cold chloroform/methanol (1:1, v/v). The resulting chloroform/methanol/water extract was processed as described in Materials and methods. Lipids were analysed by TLC (silica 60, solvent system C). A thin layer chromatogram corresponding to each sample is shown in Figure 3.

[a]Each sample was prepared by incubating 60 μl of lysate ($6 \times 10^7$ cell equivalents) with 10 μl of pre-incubation buffer[e] for 20 min at 37°C. GDP-[$^3$H]mannose ($1.93 \times 10^6$ c.p.m. in 10 μl incubation buffer) was added and the incubation was continued for an additional 10 min. The samples were then treated as follows. The reaction in sample 1 was terminated without further incubation and lipids were extracted as described above. Samples 2 and 3 were 'chased' by adding 20 μl of a solution of GDP-mannose (5 mM in incubation buffer) and incubating for 30 and 60 min respectively prior to lipid extraction. Sample 4 was 'chased' by adding 20 μl of a solution of GDP-mannose and UDP-GlcNAc (5 mM each in incubation buffer) and incubating for 60 min prior to lipid extraction.

[b]Mannosylated intermediates include lipids I, II, III and IV, as well as two minor unidentified species chromatographing in the vicinity of lipid I (see Figure 3).

[c]The reduction in intensity of $dol_t$-P-mannose in the chase samples (B, C, D) probably reflects rapid turnover, reminiscent of the catabolism of oligosaccharide pyrophosphoryldolichol lipids reported in other systems (Belard *et al.*, 1988).

[d]The loss of labelled P2 and P3 between the 30 min and 60 min chase points (B and C) is consistent with transfer of the labelled lipids to endogenous VSG polypeptide acceptors (S.Mayor, A.K.Menon and G.A.M.Cross, in preparation).

[e]Pre-incubation buffer is incubation buffer + 2 μM GDP-Man, 10 mM ATP, 10 mM CoA, 2 μg/ml tunicamycin, 80 U/ml creatine kinase, 100 mM creatine phosphate.

labelling pulse (Table IIIA and D, Figure 3A and D). In contrast, significant quantities of [$^3$H]mannose labelled P2 and P3 were synthesized in samples chased in the presence of UDP-GlcNAc (Table IIIB and C, Figure 3B and C). Since radiolabelled P2 and P3 were synthesized during the chase period, it is clear that at least one of the three mannose residues in each lipid is *not* directly donated by GDP-mannose but is, instead, derived from a radioactive precursor generated during the labelling pulse.

The pulse−chase results were taken a step further by methylation analysis of lipid-derived neutral glycans. P2 and P3 labelled during the chase period (Table III, samples B and C) were purified by TLC and neutral glycans were prepared. The identity of the radiolabelled glycans was confirmed by chromatographing aliquots on Dionex HPLC: in both cases (P2 and P3) the radioactivity eluted as a single peak corresponding to the Man$\alpha$1-2Man$\alpha$1-6Man-2,5-anhydromannitol ($Man_3$AHM) standard (data not shown). The remainder of each sample was permethylated and hydrolysed, and the resulting partially O-methylated mannose species were analysed by TLC. In both cases the chromatograms consisted of three radiolabelled species corresponding to 2,3,4-tri-,3,4,6-tri, and 2,3,4,6-tetra-*O*-methyl mannose (data not shown). These results clearly indicate that for both lipids all three mannose residues are derived from a precursor generated during the labelling pulse and *not* directly from GDP-mannose. Taken together with the dol-P stimulation and amphomycin inhibition results described above, the data suggest that the mannose donor generated during the GDP-[$^3$H]mannose labelling pulse is $dol_t$-P-[$^3$H]mannose.

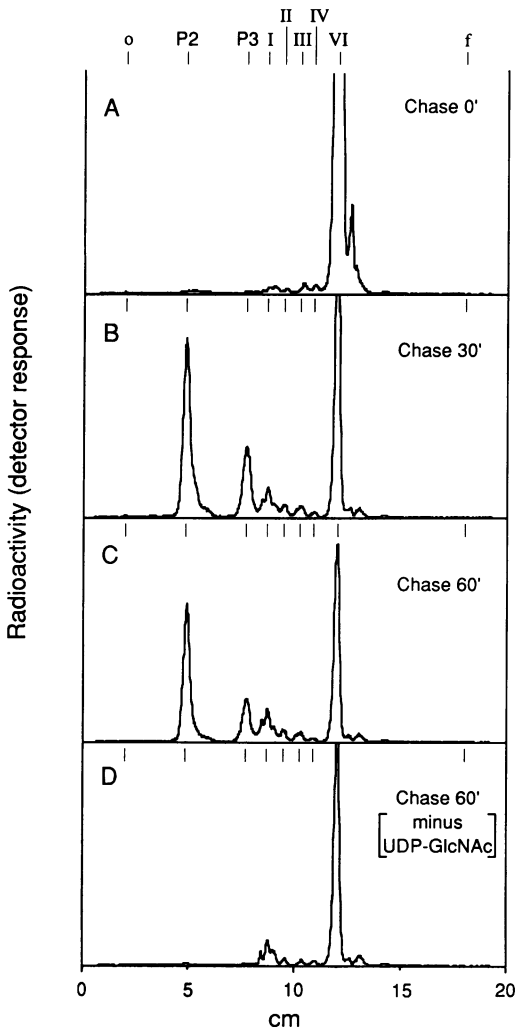


**Fig. 3.** Pulse−chase labelling of P2 and P3 *in vitro*. Lysates were pulse-labelled with GDP-[$^3$H]mannose and chased in the presence (panels B and C) or absence (panel D) of UDP-GlcNAc. Experimental details are provided in the text and in Table III. At the end of the incubation lipids were extracted into CMW and the CMW-derived butanol phase was analysed by TLC (silica 60, solvent system C). The plates were scanned for radioactivity with a Berthold LB 2842 automatic scanner. The figure shows thin layer profiles corresponding to the end of the labelling pulse (panel **A**), chase periods of 30 min (panel **B**) and 60 min (panel **C**) in the presence of UDP-GlcNAc, and a 60 min chase in the absence of UDP-GlcNAc (panel **D**). Migration positions of GPI lipids (P2, P3 and mannosylated intermediates) and lipid VI ($dol_t$-P-mannose) are indicated at the top of each panel; the lipids are defined in Table I. The extent of incorporation of radioactivity into the various lipids is summarized in Table III. The chromatograms contain two labelled products flanking lipid I which have not been previously identified; these may be dolichol-linked mannosylated species and are discussed in the text.

### *In vitro labelling of glycosyl-PI lipids via dol-P-[$^3$H]mannose*

In order to assess directly the role of dolichol-P-mannose in GPI synthesis, lysates were incubated with $dol_m$-P-[$^3$H]mannose for 40 min at 37°C in the presence of GDP-mannose and amphomycin. $Dol_m$-P-[$^3$H]mannose was added as a solution in 0.2% Triton X-100 (final concentration 0.0064%). Since trace amounts of GDP-[$^3$H]-mannose were detected in preliminary incubations with $dol_m$-P-[$^3$H]mannose, the reaction mixtures contained non-radioactive GDP-mannose (1.2 mM) to eliminate the possibility of labelling via GDP-[$^3$H]mannose, as well as amphomycin to prevent the synthesis of non-radioactive $dol_t$-P-mannose from GDP-mannose and $dol_t$ during the incubation. Appropriate controls were provided by lysates incubated with GDP-[$^3$H]mannose in the presence or absence of detergent and/or amphomycin. At the end of the incubation period the reactions were terminated by CMW extraction, and the CMW-derived butanol extracts were analysed by TLC.

Thin layer chromatograms of CMW-derived butanol extracts from control incubations with GDP-[$^3$H]mannose are shown in Figure 4A−C. The profiles show that the presence of detergent in the incubation mixture partially inhibits the fatty acid remodelling reactions (note significant amounts of unmodelled P2 and P3) and depresses the synthesis of [$^3$H]mannose labelled P2 and P3 (Figure 4A and B, Table IV A and B) without any apparent effect on the synthesis of mannosylated GPI intermediates and $dol_t$-P-mannose. The profile shown in Figure 4C (quantitation given in Table IV C) confirms that the concentration of amphomycin used (0.4 mg/ml) effectively inhibits the synthesis of $dol_t$-P-mannose (~95%) as well as of the entire spectrum of GPI lipids (>94%).

A thin layer chromatogram of the CMW-derived butanol extract from a $dol_m$-P-[$^3$H]mannose labelling experiment is shown in Figure 4D. The profile clearly shows peaks of radioactivity corresponding to the entire spectrum of previously identified mannosylated GPI intermediates (lipids I, II, III, and IV), as well as un-modelled forms of P2 and P3. With the exception of a major peak corresponding to the input radioactivity ($dol_m$-P-[$^3$H]mannose), the chromatogram is very similar to that obtained from a

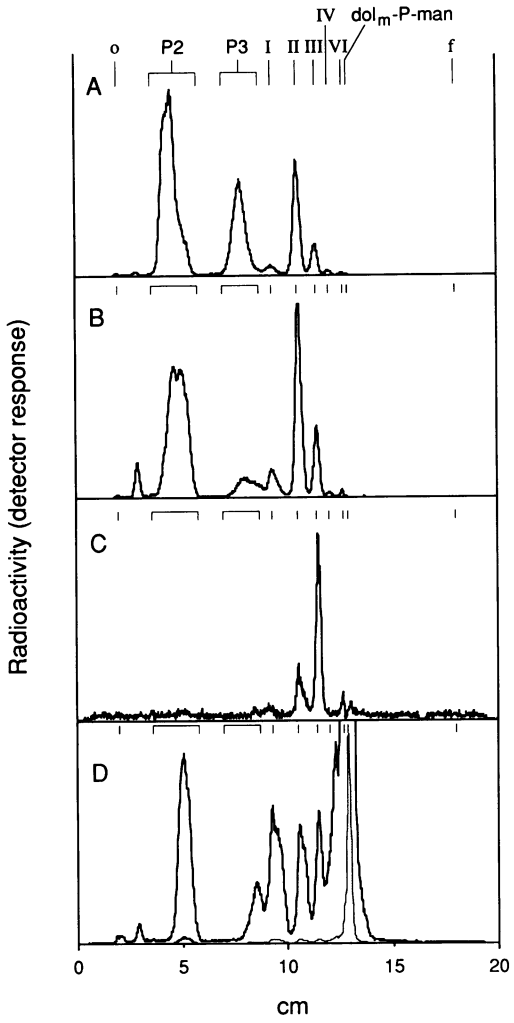


**Fig. 4.** Incubation of lysates with dolichol-P-[$^3$H]mannose results in the labelling of mannosylated glycosyl-phosphatidylinositol species. Lysates ($8 \times 10^7$ cell equivalents) were incubated with (**A**) GDP-[$^3$H]mannose ($1.63 \times 10^6$ c.p.m.), (**B**) GDP-[$^3$H]mannose ($1.63 \times 10^6$ c.p.m.) in the presence of 0.0064% Triton X-100, (**C**) GDP-[$^3$H]mannose ($1.63 \times 10^6$ c.p.m.) in the presence of 0.0064% Triton X-100 and amphomycin (0.4 mg/ml) and (**D**) dolichol$_m$-P-[$^3$H]mannose ($3.3 \times 10^5$ c.p.m.) in the presence of 0.0064% Triton X-100 and amphomycin (0.4 mg/ml). The total volume of each sample was 125 μl. After incubation for 40 min at 37°C, the reactions were terminated as described in Table IV, and lipids in the CMW-derived butanol phase were chromatographed on silica 60 TLC plates using solvent system B. Radioactivity was detected with a Berthold LB 2842 automatic scanner. Panels A–C show thin layer profiles corresponding to the three GDP-[$^3$H]mannose incubations. Panel **D** shows both an 28× amplified view (outline) and a full scale view (grey tone) of the lipid profile from a dolichol$_m$-P-[$^3$H]mannose labelling incubation. Table IV summarizes the extent of incorporation of radioactivity into each of the labelled lipids. The migration positions of lipid standards are indicated at the top of each chromatogram. The broad regions marked P2 and P3 indicate the migration of fatty acid remodelling intermediates as well as the mature dimyristyl lipids (see Table I). Lipids I, II, III, IV and VI are defined in Table I, o = origin, f = front.

GDP-[$^3$H]mannose labelling experiment suggesting that GPI lipids may be labelled *in vitro* via dol$_m$-P-[$^3$H]mannose.

Neutral glycan analysis was performed to confirm the identity of the lipids labelled via dol$_m$-P-[$^3$H]mannose. Regions of the thin layer chromatogram corresponding to authentic P2 and P3 were scraped and extracted, and neutral glycans were prepared by dephosphorylation, deamination and reduction. Dionex HPLC analysis of an aliquot of the P2 material showed a single peak corresponding to the Manα1-2Manα1-6Man-2,5-anhydromannitol ($Man_3$AHM) standard (Figure 5A), confirming the identity of dol$_m$-P-[$^3$H]mannose labelled P2. Approximately half the P3 material chromatographed as $Man_3$AHM (retention time ~13.5 min); the remainder of the radioactivity eluted as $Man_1$AHM (retention time ~6 min, 16% of the total radioactivity) and an unresolved pair of peaks running between 3.5 and 4.5 min (data not shown). The origin of the additional peaks in the P3 sample is unclear. They may be derived from dolichol-linked oligosaccharides, minor amounts of which seem to be synthesized from endogenous acceptors in this system despite the inhibition of *de novo* synthesis by tunicamycin present in the incubation mixtures.

Neutral glycans were prepared from material extracted from regions of the thin layer chromatogram (Figure 4D) corresponding to authentic lipids I, II, and III as follows. In order to eliminate anticipated contamination from the considerable dol$_m$-P-[$^3$H]mannose peak (Figure 4D), the lipids were deacylated by treatment with 30% $NH_3$/methanol (1:1, v/v) for 4 h at 37°C, dried and partitioned between water and butanol. Cleavage of dol$_m$-P-[$^3$H]mannose under these deacylation conditions was <2%. The material recovered in the aqueous phase (87%, 65% and 37% of the total radioactivity in the three samples I, II, and III respectively) was deaminated and reduced. An aliquot of each sample was analysed on Dionex HPLC. The neutral glycan preparation derived from the lipid I region of the thin layer chromatogram (Figure 4D) chromatographed as $Man_3$AHM (74% of the radioactivity) and $Man_1$AHM (26%) (data not shown). The $Man_1$AHM component unexpectedly detected in the analysis may originate from lyso lipid III which chromatographs close to lipid I (Menon *et al.*, 1990). The glycan preparation derived from the lipid II region chromatographed almost exclusively as $Man_2$AHM (Figure 5B), and the preparation from the lipid III region ran as $Man_1$AHM (68%) and $Man_3$AHM (32%) (data not shown).

The neutral glycan analyses described above confirm the identity of the dol$_m$-P-[$^3$H]mannose labelled lipids suggested by TLC. In addition, the detection of $Man_1$AHM in these analyses clearly indicates that the first mannose residue (proximal to glucosamine) in the GPI glycan can be labelled *in vitro* directly by dol$_m$-P-[$^3$H]mannose.

The butanol phases obtained after deacylation and phase separation of dol$_m$-P-[$^3$H]mannose labelled lipids I, II and III were re-chromatographed on thin layer plates (silica 60, solvent system B) to check if contaminating dol$_m$-P-[$^3$H]mannose was the only base resistant material in the three preparations. All three chromatograms showed base resistant peaks corresponding to the starting material in addition to dol$_m$-P-[$^3$H]mannose and one or more unidentified products. This spectrum of lipids may include the dolichol-linked oligosaccharides mentioned above as well as lyso lipid species.

### *All the mannose residues in glycosyl-PI lipids can be labelled via dol$_m$-P-[$^3$H]mannose*

After aliquots of the neutral glycan preparations from dol$_m$-P-[$^3$H]mannose labelled P2, lipid I and lipid II were analysed by Dionex HPLC as described above, the remainder of each sample was permethylated and hydrolysed, and the

**Table IV.** Incubation of lysates with GDP-[$^3$H]mannose or dol$_m$-P-[$^3$H]mannose: incorporation of radioactivity into glycolipids[a]

| Sample | Radiolabel | TX-100[b] (%) | Amphomycin (mg/ml) | GDP-Man (mM) | P2 | P3 | I | II (c.p.m. × $10^{-3}$) | III | IV | VI |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A | GDP-[$^3$H]Man | 0 | 0 | 0 | 220 | 99.5 | 9.45 | 61.8 | 16.0 | 2.34 | 1.05 |
| B | GDP-[$^3$H]Man | 0.0064 | 0 | 0 | 115 | 17.8 | 11.9 | 55.4 | 18.2 | 1.19 | 1.12 |
| C | GDP-[$^3$H]Man | 0.0064 | 0.4 | 0 | 0.06 | 0.02 | 0.08 | 0.27 | 0.78 | 0.07 | 0.06 |
| D | dol$_m$-P-[$^3$H]man | 0.0064 | 0.4 | 1.2 | 10.8 | 3.48 | 8.33 | 4.50 | 2.80 | 2.59 | – |

Lysates ($8 \times 10^7$ cell equivalents) were incubated with GDP-[$^3$H]mannose ($1.63 \times 10^6$ c.p.m.) or dol$_m$-P-[$^3$H]mannose ($3.3 \times 10^5$ c.p.m.) for 40 min at 37°C. The total volume of each sample was 125 μl. At the end of the incubation period, the reactions were stopped by adding 125 μl ice-cold buffer 4 and 1.7 ml ice-cold chloroform/methanol (1:1, v/v). The resulting chloroform/methanol/water (CMW) extract was processed as described in Materials and methods. Lipids were analysed by TLC. Radioactivity was quantitated as described in Figure 2. A thin layer chromatogram corresponding to each sample is shown in Figure 4.

[a]The glycolipids P2, P3, I, II, III, IV and VI are defined in Table I and in Figure 4.

[b]Triton X-100 (0.2% solution in buffer 4) was used to solubilize dol$_m$-P-[$^3$H]man (sample D) as described in Materials and methods; similar quantities of the detergent were included in control samples B and C.

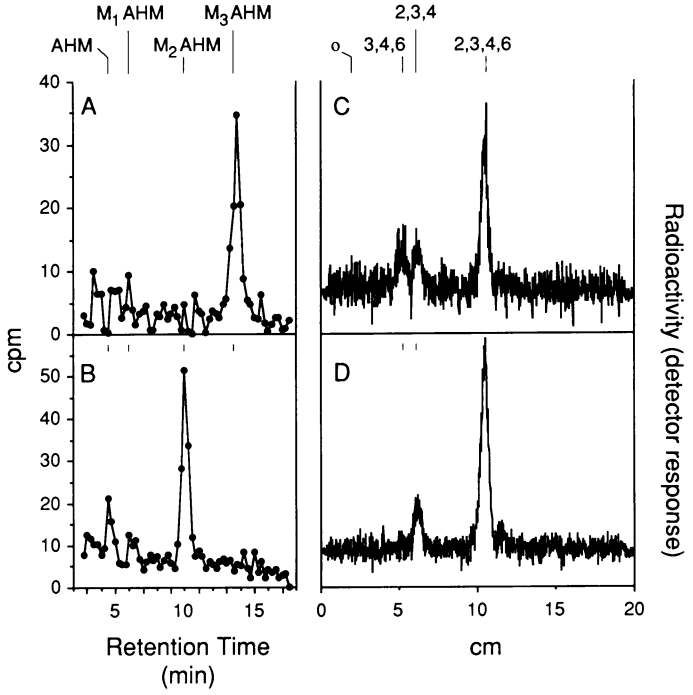


**Fig. 5.** Neutral glycan analysis of lipids labelled via dolichol$_m$-P-[$^3$H]mannose. The CMW-derived butanol extract from a dol$_m$-P-[$^3$H]mannose labelling experiment was chromatographed as shown in Figure 4D and labelled lipids corresponding to authentic P2 and lipid II were isolated by scraping and extraction. Neutral glycans were prepared as described in Materials and methods and Results. An aliquot (200–300 c.p.m.) of each sample was analysed by Dionex HPLC. Non-radioactive standards [AHM and Man$_3$AHM (M$_3$AHM)] were included in the run and detected by pulsed amperometric detection. The elution positions of the M$_1$[$^3$H]AHM (M$_1$AHM) and Man$_2$[$^3$H]AHM (M$_2$AHM) standards were determined in separate runs. The figure shows Dionex profiles of neutral glycan preparations corresponding to P2 (panel **A**) and lipid II (panel **B**). The remainder of each neutral glycan preparation was subjected to methylation analysis (panels **C** and **D**). The samples were permethylated and hydrolysed, and the resulting partially O-methylated mannose species (total recovery of radioactivity ~44–48%) were analysed by TLC (silica 60, solvent system E). Previously characterized samples of [$^3$H]mannose labelled P2 and P3 (Mayor *et al.*, 1990a) were subjected to the same analysis and chromatographed alongside. The migration positions of tri- and tetra-O-methylated mannose species derived from these samples are indicated at the top of each panel. The figure shows thin layer profiles of radiolabelled partially methylated mannose species derived from P2 (panel C, 1200 c.p.m. chromatographed) and lipid II (panel D, 1830 c.p.m. chromatographed). O = origin, front = 20 cm.

resulting partially O-methylated mannose species were analysed by TLC.

Methylation analyses of the P2 glycan [Manα1-2Manα-1-6Man-2,5-anhydromannitol (Man$_3$AHM), Figure 5A] yielded three radiolabelled products corresponding to 2,3,4-tri-, 3,4,6-tri- and 2,3,4,6-tetra-*O*-methyl mannose (Figure 5C). Similar results were obtained with the lipid I preparation (data not shown). Methylation analysis of lipid II yielded two partially O-methylated mannose species corresponding to 2,3,4-tri- and 2,3,4,6-tetra-*O*-methyl mannose (Figure 5D). The data clearly indicate that all the mannose residues in the GPI sequence Manα1-2Manα1-6-Man can be labelled *in vitro* via dol$_m$-P-[$^3$H]mannose. Taken together with the amphomycin inhibition data and the pulse–chase results presented above, the data argue strongly for a dolichol-P-mannose intermediate in the transfer of mannose from GDP-mannose to incompletely mannosylated glycosyl-PI acceptors.

## Discussion

In this paper we present evidence that the three mannose residues in the core glycan (Manα1-2Manα1-6Manα1-GlcN-inositol) of trypanosome glycosyl-phosphatidylinositol (GPI)

lipids are derived from dolichol-P-mannose. Initial data in support of this conclusion were obtained from *in vitro* labelling experiments in which trypanosome membranes were incubated with GDP-[$^3$H]mannose. The transfer of [$^3$H]mannose from GDP-[$^3$H]mannose into GPI lipids was stimulated by dolichol-phosphate and inhibited by amphomycin, a known inhibitor of dolichol-P-mannose synthesis. The synthesis of both 'P2-like' (PI-linked) and 'P3-like' (acyl PI-linked) GPI species was affected in these experiments, consistent with our previous speculation that the two types of GPI lipids are synthesized via a common biosynthetic route (Mayor *et al.*, 1990a; Menon *et al.*, 1990). Direct evidence for the involvement of dolichol-P-mannose in GPI synthesis was obtained from experiments in which lysates were incubated with dolichol-P-[$^3$H]mannose. The entire spectrum of GPI lipids previously labelled via GDP-[$^3$H]mannose could also be labelled via dolichol-P-[$^3$H]mannose. Labelling was achieved in the presence of non-radioactive GDP-mannose to rule out a labelling pathway involving the synthesis of GDP-[$^3$H]mannose from $dol_m$-P-[$^3$H]mannose. Neutral glycans were prepared from $Man_2$- and $Man_3$-containing GPI lipids labelled directly via dolichol-P-[$^3$H]mannose. Methylation analysis of the neutral glycans permitted the unambiguous identification of individual mannose residues as their corresponding partially O-methylated derivatives. In each case all the mannose residues were found to be radiolabelled, clearly indicating that all three residues were derived from dolichol-P-mannose.

In addition to confirming the involvement of dolichol-P-mannose in GPI assembly, the methylation data raise questions concerning the mechanism of mannose addition. Although the unequal intensity of the partially methylated mannose species in chromatograms such as those shown in Figure 5 (panels C and D) may be attributed to non-steady-state labelling under the assay conditions (steady-state labelling of these glycans should give peaks of equal intensity; Menon *et al.*, 1990), comparison of the Man$\alpha$1-6Man profile (Figure 5D) with the Man$\alpha$1-2Man$\alpha$1-6Man profile (Figure 5C) suggests that mannosylation reactions may occur via a 'trimming and addition' pathway involving $\alpha$-mannosidase trimming of a $Man_2$-lipid to a $Man_1$-lipid, followed by the addition of Man$\alpha$1-2Man from a dol-P-mannose-derived donor to the 6-position of the $Man_1$-GPI lipid acceptor. The complexity of the membrane preparation used and the lack of information on endogenous GPI pools rule out a firmer conclusion at this point.

The involvement of dolichol-P-mannose as a mannose donor in GPI assembly has implications for the membrane topology of the assembly process. Unlike GDP-mannose which apparently cannot be translocated into the lumen of the endoplasmic reticulum (ER) (Hirschberg and Snider, 1987; Lennarz, 1987), dolichol-P-mannose, synthesized from GDP-mannose and dolichol-phosphate on the cytoplasmic face of the ER, has been shown to participate in reactions such as the direct O-mannosylation of proteins in the lumen of yeast ER (Sharma *et al.*, 1974; Haselbeck and Tanner, 1983). In addition, topological analyses of the assembly of the lipid-linked oligosaccharide precursors of asparagine-linked sugars indicate that, while GDP-mannose dependent mannosylation steps ($GlcNAc_2$-PP-dolichol→ $Man_5GlcNAc_2$-PP-dolichol) occur on the cytoplasmic face of the ER membrane, dolichol-P-mannose dependent elongation of $Man_5GlcNAc_2$-PP-dolichol to $Man_9GlcNAc_2$-PP-dolichol occurs in the ER lumen (reviewed by Hirschberg and Snider, 1987; Lennarz, 1987). The transbilayer movement of dolichol-P-mannose indicated by these observations has been demonstrated in an artificial membrane system (Haselbeck and Tanner, 1982, 1984). GPI biosynthesis, like the biosynthesis of the dolichol-linked oligosaccharide precursors of asparagine-linked sugars, probably takes place in the membrane of the ER. In both cases, a lumenally oriented product is required for transfer to newly synthesized and translocated proteins. The availability of UDP-GlcNAc and phosphatidylinositol in the ER lumen (Hirschberg and Snider, 1987; Higgins *et al.*, 1989) is consistent with the initiation of GPI glycosylation on the lumenal face of the ER membrane. By analogy to the dolichol-P-mannose-dependent mannosylation reactions described above, we speculate that mannosylation steps in GPI assembly occur in the lumenal leaflet of the ER membrane. Recent data indicating that the terminal ethanolamine-phosphate group is most likely derived from a lipid-linked donor (A.K.Menon, S.Mayor, M.Eppinger and R.T.Schwarz) suggest that the final step in GPI assembly could also occur in the ER lumen to generate appropriately located glycolipid anchor precursor molecules *in situ*.

## Materials and methods

### *Materials*

Guanosine diphospho-[3,4-$^3$H]mannose (16.8 or 20 Ci/mmol) was purchased from Du Pont-New England Nuclear. Pig liver dolichol phosphate (16–21 isoprene units) was from Sigma Chemical Co., amphomycin was a gift from Troponwerke, Cologne (Germany), and phosphatidylinositol specific phospholipase C from *Bacillus thuringiensis* was a gift from Dr M.G.Low (Columbia University). All solvents were reagent or HPLC grade and were obtained from commercial sources.

A sample of 2,3,4,6-tetra-*O*-methyl-$\alpha\beta$-methylmannoside and a mixture of partially O-methylated methyl mannosides (2,3,4-tri- and 2,3,6-tri-*O*-methyl-$\alpha\beta$-methylmannosides) prepared as described by Hull and Turco (1985) were gifts from Dr S.J.Turco (University of Kentucky). The samples were hydrolysed in trifluoroacetic acid as described (Hull and Turco, 1985) to generate the corresponding partially O-methylated mannose species. Additional standards (2,4-di-, 3,4,6-tri- and 2,3,4,6-tetra-*O*-methyl mannose) were generated by permethylation and hydrolysis of a completely characterized, [$^{14}$C]mannose labelled isomer of $Man_5GlcNAcGlcNAcitol$ (Vijay and Perdew, 1980) provided by Dr I.K.Vijay (University of Maryland).

DEAE-cellulose (Whatman, DE-52) was converted to the acetate form by the following procedure. The resin was gently mixed with 3 vol of 1 M NaOH for 30 min, then collected on a sintered glass filter and washed to neutrality with water. The resin was then washed sequentially with 4 vol of ethanol and 4 vol of methanol and allowed to dry. The dried resin was gently stirred overnight with glacial acetic acid, collected on a sintered glass filter, washed with 99% methanol and stored in 99% methanol at 4°C.

### *Radiolabelling, extraction and analysis of lipids*

Trypanosomes of the Molteno Institute trypanozoon antigenic type 1.4 and 1.5 (variant clone 117 and 118) of *T.brucei* strain 427 were purified from infected rat blood. Two types of membrane fractions ('lysates' and 'membranes') were prepared. The term lysate will be used to refer to washed preparations of lysed trypanosomes prepared as described by Masterson *et al.* (1989); membranes will refer to the 100 000 *g* pellet obtained after differential centrifugation of lysed and homogenized trypanosomes as described by Menon *et al.* (1990).

Membranes were incubated with GDP-[$^3$H]mannose (~2 $\mu$Ci) or dol-P-[$^3$H]mannose (usually 4–6 $\mu$l of a 70 000–100 000 c.p.m./$\mu$l solution in 0.2% Triton X-100) in the presence of ATP (1 mM) and CoA (1 mM) essentially as described (Menon *et al.*, 1990). In many instances assays were supplemented with UDP-GlcNAc as indicated. The volume of the reaction mixture was ~60 $\mu$l and incubations were typically carried out for 30 min

at 37°C. Amphomycin (10 mg/ml suspension in water) and/or dolichol phosphate (4 mg/ml solution in 0.2% Triton X-100) were included in some incubations. The final concentration of detergent was maintained below 0.02%. Reactions were terminated by the addition of ice-cold chloroform/methanol (CM, 2:1, v/v) and the labelled membranes were subjected to differential solvent extraction as described (Menon *et al.*, 1990). Glycosyl-phosphatidylinositol species lacking the terminal ethanolamine-phosphate group were quantitatively (~95%) extracted into CM; the mature ethanolamine-phosphate-containing glycolipids, P2 and P3, were solubilized in a subsequent extraction step using chloroform/methanol/water (CMW, 10:10:3, v/v/v). Labelled non-lipid contaminants in the CM extract were removed by Folch-washing (Sharma *et al.*, 1974). A similar purification of the CMW extract was achieved by *n*-butanol/water partitioning of the dried extract: lipids were quantitatively extracted into the butanol phase.

Frozen lysates prepared from tunicamycin-treated trypanosomes were thawed and washed three times in ice-cold 'wash buffer' (50 mM Na-HEPES pH 7.4, 25 mM KCl, 5 mM $MgCl_2$) by resuspension (10 ml per wash) and centrifugation (Sorvall SS-34 rotor, 12 000 *g*, 10 min). The lysates were finally resuspended in 'incubation buffer' ('wash buffer' + 5 mM $MnCl_2$) at $5 \times 10^8$ cell equivalents/ml. Lysates were incubated with GDP-[$^3$H]mannose or dol-P-[$^3$H]mannose (delivered as a solution in 0.2% Triton X-100) in the presence of 1 mM ATP, 1 mM CoA and 0.2 μg/ml tunicamycin (added as a solution in 95% ethanol, final concentration of ethanol < 0.008%). UDP-GlcNAc (1 mM) was added to the incubation mixtures as indicated. The total volume of each incubation was 100–125 μl. At the end of the incubation period, the reaction volume was made up to 250 μl by adding ice-cold incubation buffer and lipids were extracted by adding 1.7 ml chloroform/methanol (1:1, v/v). The resulting CMW extract, containing all the GPI species indicated in Table I, was processed as described above.

Labelled lipids in the Folch-washed CM extracts and in the CMW extract-derived butanol phases were analysed by TLC using either Silica Si 50000 HPTLC or Silica 60 TLC plates (Merck) developed in one of the following solvent systems: A–chloroform/methanol/acetic acid/water (25:15:4:2); B–chloroform/methanol/water (10:10:2.5); C–chloroform/methanol/water (10:10:2.7); D–chloroform/methanol/30% ammonia/1 M ammonium acetate/water (180:140:9:8:23). The plates were scanned for radioactivity with a Berthold LB 2842 automatic scanner and lipids were purified by scraping and extraction as described (Menon *et al.*, 1990).

Neutral glycans were prepared by deamination and reduction of TLC-purified lipids. In some instances the lipids were dephosphorylated by treatment with ice-cold aqueous HF prior to deamination and reduction. For other analyses, lipids were deacylated by treatment with 30% $NH_3$/methanol (1:1, v/v) for 4 h at 37°C, dried, partitioned between water and butanol, and the glycerol-linked glycans in the aqueous phase were deaminated and reduced.

Desalted neutral glycans were analysed by anion exchange chromatography on a Dionex Basic Chromatography System (Dionex Corporation, Sunnyvale, CA) using the following gradient elution program: 100% Solution A (100 mM NaOH) for 3 min after sample injection, then a linear gradient to 40% Solution A, 60% Solution B (100 mM NaOH, 0.5 M $CH_3COONa$) at 33 min. The flow rate was 1 ml/min. Fractions (0.25 min) were taken for liquid scintillation counting.

Neutral glycans were also subjected to methylation analysis. Methylation was performed by the method of Ciucanu and Kerek (1984), and the permethylated glycans were hydrolysed in 2 N trifluoroacetic acid for 4 h at 120°C. The resulting partially O-methylated mannose species were analysed by TLC on silica 60 using solvent system E–benzene/acetone/water/30% ammonia (50:200:3:1.5). Detailed procedures for neutral glycan preparation, Dionex HPLC and methylation analysis are given elsewhere (Mayor *et al.*, 1990a; Menon *et al.*, 1990).

### *Preparation of dolichol-P-[$^3$H]mannose*

Dolichol$_m$-P-[$^3$H]mannose was prepared by incubating membranes from chick embryo fibroblasts with GDP-[$^3$H]mannose and pig liver dolichol phosphate (dol$_m$-P) according to published procedures (Krag and Robbins, 1977; Schwarz and Datema, 1982). Chick embryo fibroblasts [20 Petri dishes (9–14 cm diameter), 3–7 day cultures] were dislodged from the Petri dishes, swollen in a hypotonic buffer and homogenized in a Dounce homogenizer (12 strokes). The cell homogenate was subjected to differential centrifugation as described (Schwarz and Datema, 1982) and the high speed membrane pellet was resuspended in 0.5–1 ml assay buffer (Schwarz and Datema, 1982) and added to assay tubes (50 μl membranes/tube) containing GDP-[$^3$H]mannose (2–2.5 μCi) and dol$_m$-P (6 μl, 4 mg/ml in 0.2% Triton X-100). After incubation for 5 min at 37°C, the reaction was stopped by the addition of 1.5 ml chloroform/methanol (CM, 2:1, v/v). The membranes were pelleted by mild centrifugation and the membrane pellet was re-extracted with 0.5 ml CM. The CM extracts were pooled, Folch-washed, then dried using a Speed-Vac evaporator (Savant Instruments, Inc.). The dried residue was resuspended in water-saturated *n*-butanol (~500 μl) and applied to a 3 ml column of DEAE-cellulose (acetate form) equilibrated in 99% methanol. After loading the sample, the column was washed with 99% methanol (15 ml), prior to elution of dol$_m$-P-[$^3$H]mannose with 0.2 M ammonium acetate (in 99% methanol). Fractions (0.5 ml) were collected during the elution step and an aliquot (5 μl) of each fraction was taken for liquid scintillation counting. Chloroform was added to the peak fractions to bring the composition to 2:1 (chloroform/methanol, v/v) and the fractions were subjected to Folch washing. The Folch-washed samples were dried, resuspended in a small volume of CM and further purified by TLC on silica 60 plates using solvent system B. The region of the plate corresponding to the major peak (>95% of the radioactivity) [relative migration ($R_f$) ~0.75] was scraped and extracted twice with CMW. The CMW extract was dried and partitioned between water and butanol. Thin layer analysis of a small aliquot of the butanol phase showed a single peak corresponding to dol$_m$-P-mannose. The yield of radiolabelled dol$_m$-P-[$^3$H]mannose was $0.5–1 \times 10^7$ c.p.m. per 20 Petri dishes of cells.

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